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Characterization of *Toxoplasma gondii* from Raccoons (*Procyon lotor*), Coyotes (*Canis latrans*), and Striped Skunks (*Mephitis mephitis*) in Wisconsin Identified Several Atypical Genotypes

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ABSTRACT: During 2005–2006, sera and tissues from raccoons (*Procyon lotor*), coyotes (*Canis latrans*), and skunks (*Mephitis mephitis*) from the state of Wisconsin were tested for *Toxoplasma gondii* infection. Antibodies to *T. gondii* were found in 32 of 54 (59.2%) raccoons, 18 of 35 (51.4%) coyotes, and 5 of 7 (71.4%) skunks using the modified agglutination test and a cut-off titer of 1:20. Pooled tissues (brains, hearts, and tongues) from 30 raccoons, 15 coyotes, and 1 skunk were bioassayed for *T. gondii* infection in mice or cats. Viable *T. gondii* was isolated from 5 of 30 (16.7%) raccoons, 6 of 15 (40.0%) coyotes, and the skunk. Genetic characterization of the 12 parasite isolates by multilocus PCR-RFLP markers revealed 6 different genotypes including 5 atypical and 1 archetypal II lineages. The results indicate the prevalence of *T. gondii* in wildlife mammals is high and that these animals may serve as an important reservoir for transmission of *T. gondii*.

Toxoplasma gondii isolates have been classified into 3 genetic Types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997). Until recently most isolates of *T. gondii* were considered clonal, with little genetic diversity (Lehmann et al., 2006); however, most of this information is derived from isolates from domestic animals and human patients. Little is known of the prevalence and distribution of genotypes of *T. gondii* in wildlife species (Dubey, Graham et al., 2002; Dubey, Parnell et al., 2004). In limited studies, all isolates of *T. gondii* from white-tailed deer were found to be Type II at the SAG2 locus (Dubey, Graham et al., 2004). Among marine mammals, viable *T. gondii* has been isolated from sea otters (Cole et al., 2000; Lindsay et al., 2001; Miller et al., 2001; Conrad et al., 2005), a Pacific harbor seal (Miller et al., 2001), and a California sea lion (Conrad et al., 2005). These isolates from marine mammals have been PCR-RFLP genotyped into 2 groups, including the archetypal II and a new type named ''genotype x,'' with the latter being the predominant type in sea otters (Miller et al., 2004; Conrad et al., 2005). These results are in contrast with the Type II genotype that is widespread in domestic animals and humans throughout North America and Europe. To further investigate the prevalence and genetic diversity of *T. gondii* in wildlife, we studied the genetic and biologic characteristics of isolates of *T. gondii* from striped skunks (*Mephitis mephtis*), coyotes (*Canis latrans*), and raccoons (*Procyon lotor*) from south-central Wisconsin.

As part of a larger study to evaluate potential chronic wasting disease infection, we collected raccoons, skunks, and coyotes from a 350 km^2 area in Dane and Iowa counties (43′05″N and 89′50″W) of south-central Wisconsin from October to March 2005–2006. The landscape in this area is characterized by rolling hills and small stream valleys, with a mixture of dairy farms and oak-hickory woodlots, almost exclusively in private ownership. We obtained animal carcasses opportunistically from road kills, collaborating trappers, hunters, and using box traps. Sampling was neither random nor uniform across the study area, but primarily based on the distribution of trapper effort. Data recorded for each animal included a unique ID, geographic location, date collected, date processed, and prosector. Carcasses were refrigerated at 4 C until dissected for tissue sampling. Serum or body fluids and tissues (brains, hearts, and tongues) were sent cold from Madison, Wisconsin, to Beltsville, Maryland, for *T. gondii* examination. In total, 59 raccoons, 40 coyotes, and 7 skunks were examined in the present study (Table I).

Sera of carnivores were tested for *T. gondii* antibodies using 2-fold dilutions from 1:20 to 1:640 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Tissues of 40 animals with titers of 1:20 or higher and 6 animals (5 raccoons, 1 coyote) without serum were bioassayed for *T. gondii* infection (7 in cats and 39 in mice) after results of serologic examination were available (Table I). For bioassay in mice, 50 g of pooled brain, heart, and tongue were homogenized, digested in acidic pepsin, neutralized, and washed; the homogenate was inoculated subcutaneously into 4 out-bred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, as described (Dubey, 1998) (Table II). Imprints of lungs or brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on days 40–42 postinoculation (PI), and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 6 wk PI, and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Pooled tissues of 7 raccoons with titers of 1:20 or higher (2 raccoons; 1:80, 2 raccoons; 1:40, 1:320, 1:160 in 1 raccoon each) were fed to 1 *T. gondii*–free cat each. Feces of inoculated cats were examined for shedding of *T. gondii* oocysts 3–14 days postingestion of carnivore tissues as previously described (Dubey et al., 2002). Fecal floats were incubated in 2% sulfuric acid for 1 wk at room temperature on a shaker to allow sporulation of oocysts and were bioassayed orally in mice (Dubey and Beattie, 1988). Mice fed fecal floats were killed when they became ill or died after being fed oocysts. Their mesenteric lymph nodes were examined for tachyzoites, then homogenized in saline and inoculated into Swiss Webster mice.

Toxoplasma gondii DNA was extracted from the tissues of all infected mice from each group, and strain typing was initially performed using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB, and

TABLE I. Serology and isolation of *T. gondii* from wild mammals collected in south-central Wisconsin during 2005–2006.

Species	Total no.	Serum tested	No. seropositive	Animals bioassayed	*T. gondii* isolated
Raccoon (*Procyon lotor*)	59	54	32	30*	5
Coyote (*Canis latrans*)	40	35	18	15	6
Skunk (*Mephitis mephitis*)	7	7	5	1	1

* Tissues of 7 raccoons were bioassayed in cats, and the rest were bioassayed in mice.

TABLE II. Isolation of *T. gondii* from wild mammals collected in south-central Wisconsin during 2005–2006.

Host and I.D. (Lab. no.)	Location (county)	Longitude, latitude	MAT titer	Bioassay in cats or mice	No. of mice positive for *T. gondii**	Strain designation
Raccoon 360 (5)	Dane	89°44′54″ 43°12′07″	320	Cat†	Not done	TgRaW 1
Raccoon 361 (6)	Dane	89°44′54″ 43°12′06″	40	Cat	Not done	TgRaW 2
Raccoon 372 (17)	Dane	89°43′43″ 43°10′46″	No serum	Mice	4‡	TgRaW 3
Raccoon 378 (23)	Dane	89°43′23″ 43°10′52″	80	Mice	3	TgRaW 4
Raccoon 418 (82)	Dane	89°43′20″ 43°10′56″	40	Mice	2	TgRaW 5
Coyote 325 (31)	Iowa	89°55′58″ 43°01′27″	No serum	Mice	4	TgCyW 1
Coyote 335 (50)	Iowa	89°56′16″ 43°00′59″	40	Mice	1	TgCyW 2
Coyote 339 (65)	Dane	89°46′00″ 43°10′59″	80	Mice	5	TgCyW 3
Coyote 353 (90)	Dane	89°42′13″ 43°11′07″	80	Mice	2	TgCyW 4
Coyote 356 (95)	Dane	89°42′14″ 43°11′20″	160	Mice	1	TgCyW 5
Coyote 359 (98)	Iowa	89°53′19″ 43°10′20″	40	Mice	1§	TgCyW 6
Skunk 359 (102)	Dane	89°41′54″ 43°10′07″	80	Mice	2	TgSkW 1

* Of 5 mice inoculated.
† Cat-shed *T. gondii* oocysts.
‡ Mice died days 30, 30, 30, 31 PI.
§ Mouse died day 32 PI.

GRA6 (Dubey et al., 2006). One or 2 representative DNA extracts from mice infected with the same animal sample were genotyped with 6 additional genetic markers including c22-8, c29-2, L358, PK1, a new SAG2, and Apico to further identify isolates with high resolution (Su et al., 2006; Dubey et al., 2007) by the same method described above. Allele types for all isolates were determined based on the RFLP patterns of 6 reference strains including RH88, PTG, CTG, COUGAR, MAS, and TgCatBr5 (Su et al., 2006). These reference strains allow us to capture most known alleles for each marker and to identify potential unique alleles in new samples.

Antibodies to *T. gondii* were found in 32 of 54 (59.3%) raccoons, with titers of 1:20 in 12, 1:40 in 8, 1:80 in 4, 1:160 in 4, 1:320 in 3, and 1:640 or higher in 1. Antibodies to *T. gondii* were found in 18 of 35 (51.4%) coyotes, with titers of 1:20 in 1, 1:40 in 7, 1:80 in 6, 1:160 in 3, and 1:320 in 1. Antibodies to *T. gondii* were found in 5 of 7 (71.4%) skunks, with titers of 1:40 in 1, 1:80 in 1, 1:320 in 2, and 1:640 or higher in 1.

Toxoplasma gondii was isolated from 5 raccoons, 6 coyotes, and 1 skunk (Table II); all 12 animals were adults. Two of the 5 isolates from raccoons were obtained by bioassay in cats. The 2 cats fed tissues from raccoons 360 and 361 shed oocysts. The mice fed sporulated oocysts from these isolates became ill 11 days PI, and tachyzoites were found in their mesenteric lymph nodes. The mice inoculated with homogenate of mesenteric lymph nodes from raccoon 5 remained asymptomatic, and tissue cysts were found in their brains. The mice inoculated with mesenteric lymph homogenate of raccoon 6 died 19 days PI, and tachyzoites were found in their lungs.

Genotyping of the 12 *T. gondii* isolates identified 6 different genotypic groups (Tables II, III). Three isolates, including TgRaW1, TgCyW4, and TgCyW5, have Type II alleles at all 11 marker loci and, therefore, belong to the archetypal II lineage. Isolates TgCyW3 and TgSkW1 have Type II alleles at 10 loci, except a Type I allele at locus Apico. Isolates TgRaW5, TgCyW1, and TgCyW2 have Type II alleles at 9 loci, except Type I alleles at loci L358 and Apico. Isolates TgRaW2 and TgRaW3 have a combination of alleles I, II, and III at different loci. Isolates TgRaW4 and TgCyW6 each has a combination of alleles I and III at different loci and belong to 2 different genotypes.

Seroprevalence of *T. gondii* in raccoons in the United States is high (for review see Dubey and Odening, 2001; Hancock et al., 2005). Using a cutoff value of 1:25 in MAT, antibodies were found in 48% to 70% of raccoons from the United States (Dubey et al., 1992; Brillhart et al., 1994; Dubey et al., 1995; Hill et al., 1998; Mitchell et al., 1999; Lindsay

TABLE III. Genotyping of *T. gondii* isolates from wildlife from south-central Wisconsin during 2005–2006.

	Markers										
T. gondii strains	SAG 1	(5′ + 3′) SAG2*	SAG2†	SAG3	BTUB	GRA6	c22-8	C29-2	L358	PK1	Apico
TgRaW1	II or III	II	II	II	II	II	II	II	II	II	II
TgCyW4											
TgCyW5											
TgRaW2	I	III	III	III	III	II	I	I	I	I	I
TgRaW3											
TgRaW4	I	III	III	III	III	III	III	III	III	III	I
TgRaW5	II or III	II	II	II	II	II	II	II	I	II	I
TgCyW1											
TgCyW2											
TgCyW3	II or III	II	II	II	II	II	II	II	II	II	I
TgSkW1											
TgCyW6	I	III	III	III	III	III	III	I	III	III	III

* The SAG2 marker based on 5′- and 3′-end DNA sequence polymorphisms of SAG2 gene (Howe et al., 1997).
† The SAG2 marker developed recently based on 5′-end DNA sequence of SAG2 gene is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).

et al., 2001; Dubey et al., 2002; Hancock et al., 2005; Mitchell et al., 2006). In the present study *T. gondii* antibodies were found in 32 of 54 (59.2%) raccoons from Wisconsin, and *T. gondii* was isolated from 5 of the 30 raccoons bioassayed. The only previous report of the isolation of *T. gondii* was from 7 of 33 raccoons from Georgia (Dubey et al., 2002).

In the present study, *T. gondii* antibodies were found in 18 of 35 (51.4%) coyotes, which is similar to the 62% seroprevalence in coyotes from Texas (Lindsay et al., 1996). *Toxoplasma gondii* was isolated from the tissues of 6 coyotes from Wisconsin. The only previous report was recovery of viable *T. gondii* from tissues of 1 of 1 coyote bioassayed; this coyote was from Georgia and had a MAT titer of 1:200 (Dubey et al., 2004). Coyotes are considered resistant species to clinical toxoplasmosis, and we are not aware of any report of a clinical case of toxoplasmosis in this animal. Laboratory-reared coyotes fed *T. gondii* oocysts or tissue cysts became infected but remained asymptomatic (Dubey, 1982).

As of yet, there is no clinical report of toxoplasmosis in skunks, although antibodies have been found in skunks surveyed from Canada and the United States (Quinn et al., 1976; Schowalter et al., 1980; Dubey et al., 1995; Smith and Frenkel, 1995; Hill et al., 1998; Mitchell et al., 2006), and viable parasites were isolated from 3 of 6 skunks from Mississippi (Dubey et al., 2004). *Toxoplasma gondii* was isolated from tissues of 1 of 1 skunk from Wisconsin.

The low isolation (12 of 46 animals bioassayed) of *T. gondii* from animals in the present study was most likely related to the samples tested; it is likely that an unknown number of tissues had been exposed to freezing temperatures. Freezing is deleterious to *T. gondii* in tissues, as overnight storage in a household freezer kills tissue cysts (Dubey, 1974; Kotula et al., 1991).

Identification of 6 different genotypic groups among 12 wild animals from a small geographic area would suggest a high diversity of *T. gondii* among wildlife in Wisconsin. Additional genotypes are expected to be identified if more samples are available or if samples are collected from a larger area. Three genotypic groups ([TgRaW1, TgCyW4, and TgCyW5]; [TgCyW3 and TgSkW1]; [TgRaW5, TgCyW1, and TgCyW2]) differ from each other at only 1 or 2 loci, suggesting they are closely related, possibly from genetic recombination. Alleles from 3 major genotypes (I, II, II) were seen in 2 strains (RaW2 and RaW3), but most of the strains have combinations of alleles from just I and II, or I and III. Of the 6 genotypic groups, 5 have the combination of alleles I, II, or III at different loci, and they are either recombinant or atypical lineages, which can be distinguished only by DNA sequencing. In the present study genotyping results from (5′ + 3′) SAG2 (Howe et al., 1997) and the SAG2 (Su et al., 2006) are in agreement with each other. Identification of the widely distributed Type II lineage from wildlife in this study is not surprising; however, why the Type II lineage is predominant in human and domestic animal infections remains to be elucidated.

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Prevalence of *Toxoplasma gondii* Antibodies and the Relation to Risk Factors in Cats of Colima, Mexico

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ABSTRACT: The prevalence of *Toxoplasma gondii* antibodies in 80 domestic cats was studied in the city of Colima, Mexico, using an indirect IgG-ELISA. Antibodies were found in 28.8% of the cats, with significantly higher ($P = 0.029$) prevalence in southern and central zones (33.8%) than the northern zone (6.6%). Prevalence among cats fed with homemade food was higher than those eating commercial pellets (40.6% [vs.] 20.8%; $P = 0.055$). Overall, the prevalence of *T. gondii* antibodies in the cats of Colima was lower than in many other countries.

Toxoplasma gondii has the ability to infect a wide range of intermediate hosts, including many mammals and birds. Infection may be acquired by ingestion of food or water contaminated with oocysts released in the feces of cats, which may persist in the environment for long periods, especially in warm, humid zones (Dubey, 2004). Thus, pet and feral cats are important in transmission to humans and may cause high prevalence and outbreaks (Eng et al., 1999; Bahia-Oliveira et al., 2003; Palanisamy et al., 2006). The city of Colima, capital of the state with the same name, is located on the Pacific coast of Mexico 103°43′N, 19°15′W, and 490 m above sea level. Mean annual temperature and humidity in Colima are 25.3 C and 67%, respectively.

High seroprevalence of *T. gondii* has been reported in humans in the state of Colima (Velasco-Castrejón et al., 1992). In the present study, seroprevalence of *T. gondii* was determined in pet cats in Colima, which has a population of approximately 8,400 domestic felines. A random sample size of 80 animals was then calculated estimating 20% prevalence, 99.9% confidence, and a 5.0% error level. Because there are marked socioeconomic differences between the northern, central, and southern regions of the city, a cartographic map was used to randomly select 27 cats per zone. Nevertheless, only 15 cats could be sampled in the northern zone, due to noncompliance of pet owners. All those who consented, answered a questionnaire regarding factors related to feline toxoplasmosis, including race, gender, age, type of food provided, i.e., commercial pellets (vs.) homemade food, general health care regime (regular vaccination and antiparasite treatments), and geographic zone. Under light anesthesia using an intramuscular injection with acetopramacine and ketamine (0.1 mg/kg and 0.7 mg/kg body weight, respectively), 5 ml of blood was taken from each animal. After clotting, the serum was separated and stored at −20 C until used.

An indirect ELISA against a saline *T. gondii* RH strain crude antigen was adapted from a previously standardized technique used for rabbits (Figueroa-Castillo et al., 2006). Using pre- and postexperimental infection samples, optimal laboratory conditions were determined for this cat assay, which differed from those of rabbits in the following ways: (1) the dilution of serum (1:400); (2) the use of locally produced biotinylated anti-cat IgG at 8 μg/ml in PBS–0.05% Tween 20; (3) incubation overnight at 4 C; and (4) inclusion of a streptavidin–peroxidase conjugate, diluted 1:2,000 and left for 2 hr at 37 C. The reaction was developed with orthophenylendiamine/H_2O_2, and read at 492 nm. Some serum samples were tested by means of a western blot similar to that employed for humans, but adapted for cats (Vela-Amieva et al., 2005); the same ELISA conjugates developed by 1,4-chloro–1-naphthol/H_2O_2 were used. An ELISA cutoff of 0.32 was calculated from the mean plus 3 standard deviations of values obtained with samples from 15 cats with low probability of *T. gondii* infection, and negative by western blot. Sample size calculation and statistical analysis were performed using the Epi-INFO-2002 software of the Centers for Disease Control and Prevention, Atlanta, Georgia. The association of factors addressed with seropositivity was determined by odds ratio (OR), with 95% confidence intervals (CI), considering a $P < 0.05$ as significant by χ^2 or Fisher exact test (Rosner, 1998).

Antibodies to *T. gondii* were found in 28.8% of cats. Seroprevalence in southern and central zones (33.8%) was significantly higher than in the northern zone (6.6%), probably because of poorer socio-economic conditions in the south-central part of the city (Table I). Because only 15 cat owners in the northern zone allowed their pets to be sampled, the confidence interval observed was wide. As expected, those animals

TABLE I. Analysis of factors associated with seropositivity in pet cats of Colima, Mexico.

Variable	n	Frequency of positive (%)	OR	$CI_{95\%}$	*P*
City zone					
Center/south	65	33.8	7.16	0.88–155.32	0.029†
North	15	6.6			
Food source					
Homemade	32	40.6	2.60	0.87–7.90	0.055‡
Commercial pellets	48	20.8			
Breed					
Other	71	29.5	1.47	0.25–11.23	0.491†
Siamese	9	22.2			
Health care*					
None	40	29.6	1.16	0.34–4.02	0.787‡
Yes	26	26.0			
Age					
Adult	48	31.2	1.36	0.45–4.21	0.545‡
Juvenile	32	25.0			
Gender					
Female	39	30.0	1.21	0.41–3.57	0.697‡
Male	41	26.8			
Use of sandbox					
No	72	29.0	1.24	0.20–9.69	0.583†
Yes	8	25.0			

* Habitual administration of vaccines and antiparasite regimens.
† Fisher exact test.
‡ χ^2 test.

fed with homemade food, which included raw meat and leftovers, presented a higher prevalence of antibodies than those fed with commercial pellets. Other risks for *T. gondii* infection in cats were not significantly associated with the data generated (Table I).

Although there are several studies on *T. gondii* infection in humans and other animals in Mexico (Varela et al., 1961; Galvan Ramirez et al., 1999; Dubey et al., 2004; Galvan Ramirez et al., 2005; Figueroa-Castillo et al., 2006), little is known regarding the epidemiology of this parasite in Mexico. Varela et al. (1961) found dye-test antibodies in 52.2% of cats in Mexico, but provided no details regarding the cats. Galvan Ramirez et al. (1999) reported that 64% of 59 cat owners and 70.8% of their cats in Guadalajara had *T. gondii* antibodies. *Toxoplasma gondii* oocysts were found in the feces of 13 of 200 cats from Mexico City (de Aluja and Aguilar, 1977). For epidemiologic studies, serologic surveys in cats are more useful than the detection of oocysts because at any given time only 1% of cats are shedding oocysts in their feces (Dubey, 2004). In the present study, 28.8% of cats were seropositive for *T. gondii* and they are likely to have shed oocysts and contaminated the environment. Given that feral cats were not studied in the present work, the actual prevalence for the whole feline population might be higher.

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